## REMARKS

This is a complete response to the Final Office Action dated March 18, 2008, which is filed with a Request for Continued Examination submitted herewith.

The Final Office Action rejects claims 48-59. In an attempt to further prosecution, Applicants have amended independent claim 48. Claims 25-47 have been canceled without prejudice or disclaimer as being drawn to a non-elected group; Applicants reserve the right to pursue the original scope of the claims, such as through continuation practice.

New claims 60-69 have been added. Support for the new claims can be found, for example, in paragraphs [0029], [0032]-[0034] and [0063] as well as Examples 3 and 4. No new matter has been added.

In light of the amendments and remarks herein, Applicants believe all claims are now in condition for allowance.

### *Summary of Telephonic Interview*

Applicants' representatives, Charlton Shen and Kelly Morgan, thank Examiner Qian for the courtesy of a phone interview on July 22, 2008. During the interview, pending claim 48, potential amendments to claim 48 and prior art (Glorioso et al. and Bartholomew et al.) were discussed. Agreement as to the final disposition of the claims was not reached.

### *Amendments to the Claims:*

Though Applicants believe that the previously presented form of claim 48 is patentable over the cited art of record, the claim amendments herein are presented solely for the purpose of expediting prosecution of the present application. Such amendments are in no way to be construed as a representation of the propriety of the pending rejections. Indeed, Applicants maintain the right to prosecute any former form of the claims prosecuted in the present application, without prejudice, in a related continuing application.

Applicants have amended claim 48. No new subject matter has been added. Support for the amendment of claim 48 can be found throughout the specification, specifically in paragraphs [0007], [0008], [0060] and [0115] as well as in Examples 5-8 of the application as filed.

Claim 48 is amended to include the recitations of comprising a biocompatible "*substrate having a length in a range of about 10 cm to about 30 cm*" and a genetically altered chondrocyte "*cultured and*" modified to express a therapeutic agent in a target region associated with a disorder "*,the genetically altered chondrocyte not used for tissue repair or construction,*"...

***The Invention***

The instant invention of amended claim 48 recites a composition comprising a biocompatible substrate and a genetically altered chondrocyte. The genetically altered chondrocyte is capable of expressing a therapeutic agent in a target region associated with a disorder. Amended claim 48 recites the biocompatible substrate is *of a length in a range of about 10 cm to about 30 cm* and the genetically altered chondrocyte is *not used for tissue repair or construction*. Thus the genetically altered chondrocyte functions to deliver the therapeutic agent at the ectopic site instead of the normal function which is to repair or replace surrounding tissue. Additionally, the biocompatible substrate size recitation reinforces the utility of the composition at an *ectopic site* where the altered chondrocyte is not used for *tissue repair or construction*.

None of the cited references disclose the claimed composition either individually or in combination. Therefore, amended claim 48 and all its dependent claims are novel over the cited references.

***Novelty***

Claims 48-51 and 54-56 are rejected by the Examiner under 35 U.S.C. 102(b) as being anticipated by Glorioso et. al.'s U.S. Patent No. 6,413,511.

Glorioso et al. teach a method for introducing a polypeptide into either chondrocytes or synovial cells for *alleviating pathologies of the joint*. In fact, the main focus of Glorioso et. al. is

on the use of polypeptide introduced chondrocytes for the treatment of *joint pathologies*. Nowhere do Glorioso et al. disclose compositions comprising a *biocompatible substrate of a length in a range of about 10 cm to about 30 cm* and the genetically altered chondrocyte that *is not used for tissue repair or construction*, much less compositions wherein the genetically altered chondrocyte expresses a therapeutic agent in an *ectopic site*.

To anticipate a claim, each and every element of the claim must be found in a single prior art reference. Amended claim 48 contains several recitations that are not present in Glorioso et al., therefore the reference fails to anticipate.

Furthermore, since claims 49-51 and 54-56 are dependent upon amended claim 48, they are also not anticipated for at least the same reasons as amended claim 48. Applicants' respectfully request reconsideration of the 102 rejections.

***Non-Obviousness***

*6,413,511 in view of Bartholomew et al.*

Claims 52 and 53 are rejected by the Examiner under 35 U.S.C. 103(a) as being unpatentable over Glorioso et al. (U.S. Patent No. 6,413,511), in view of the article by Bartholomew et al.

Bartholomew et al. disclose the use of immunoisolatory devices (IID's) for aiding modified *mesenchymal stem cells* expressing human erythropoietin (EPO) after implantation into baboons. There is no teaching or suggestion in Bartholomew et al. of a composition using a *biocompatible substrate* in their implants, let alone a biocompatible substrate *of a length in a range of about 10 cm to about 30 cm*. Nor is there any teaching of a genetically altered *chondrocyte*, much less the ability of the altered cultured chondrocyte to express the therapeutic agent when delivered at an ectopic site and not used for tissue repair or construction.

The combination of Bartholomew et al. with Glorioso et al. does not render the present invention obvious. Neither Glorioso et al. or Bartholomew et al. teach of a composition with a *biocompatible substrate*, let alone a biocompatible substrate *of a length in a range of about 10 cm to about 30 cm*. Nor is it obvious to one of ordinary skill in the art to add a biocompatible

substrate to the combination since the implants of Glorioso et al. do not mention the use of it or require it to function and Bartholomew et al. utilize IID's to encompass their implants.

Furthermore, the combination of Glorioso et al. and Bartholomew et al. do not teach a *genetically altered chondrocyte,* cultured and modified to express a therapeutic agent. The cells used in Bartholomew et al. are *mesenchymal stem cells*, whereas in the current application, a *chondrocyte* is genetically altered to express a therapeutic agent. Stem cells behave, interact and respond differently than differentiated cells, such as chondrocytes.

Such differences in behavior between stem cells and differentiated cells are documented in Wutz and Jaenisch, Molecular Cell, vol. 5 pg 695 (a copy of which is appended herewith). On page 701, second paragraph, the authors describe that the induction of *Xist* transgene expression "resulted in cell death" in differentiated clones versus "no obvious effect on proliferation" in embryonic stem cells. Similarly, Niemeyer et al. (Current Stem Cell Research and Therapy, vol. 1, pg. 21) state in the first paragraph, page 22, "in contrast to chondrocytes, MSC have high expansion potential", further indicating large differences between stem cells and differentiated cells.

Thus applications and techniques performed on a stem cell, such as those taught by Bartholomew et al., are not predictably applicable or obvious for use with polypeptide introduced chondrocytes, as in Glorioso et al. Accordingly, combining the teachings of Bartholomew et al. with the polypeptide introduced chondrocytes of Glorioso et al. is not obvious to one of ordinary skill in the art. It is only with the disclosure of the present application that a skilled artisan would practice the production of a composition having a genetically altered chondrocyte cultured and modified to express an Erythropoietin protein or Erythropoietin mimetibody in accord with claims 52 or 53, respectively. Claims 52 and 53 are patentable.

*6,413,511 in view of Okada et al.*

Claims 57-59 are rejected by the Examiner under 35 U.S.C. 103(a) as being unpatentable over Glorioso, in view of the article by Okada (Biol. Pharm. Bull. 1997, Vol. 20, No. 3, p 255-258).

Okada et al. disclose encapsulating SK2 hybridoma cells that secrete anti-hIL6 monoclonal antibodies to suppress IgG1 plasmocytosis in transgenic mice. There is no teaching or suggestion in Okada et al. of using a *genetically altered chondrocyte* in their implants, much less a composition comprising a biocompatible substrate *of a length in a range of about 10 cm to about 30 cm* and a *genetically altered chondrocyte* that expresses a therapeutic agent at an ectopic site.

Similar to the response above, SK2 hybridoma cells are a very different cell type than *chondrocytes*. SK2 hybridoma cells were made from an anti-hIL-6 antibody secreting B cell that was fused with a myeloma cell to produce the anti-hIL-6 hybridoma cell line. Applications and techniques performed on a hybridoma cell line, such as those taught by Okada et al., are not obvious or applicable to polypeptide introduced chondrocytes, as in Glorioso et al. Thus combining the teachings of Okada et al. with the polypeptide introduced chondrocytes of Glorioso et al. is not obvious to one of ordinary skill in the art.

In addition, Okada et al. fail to remedy the deficiencies of Glorioso et al. by failing to teach a biocompatible substrate *of a length in a range of about 10 cm to about 30 cm.* While Okada et al. do utilize a biocompatible substrate in their implant, they provide no guidance on a composition comprising a biocompatible substrate *of a length in a range of about 10 cm to about 30 cm.*

In light of the remarks above, amended claim 48 is patentable and distinct over the combination of Glorioso et al. in view of Okada et al. Claims 57-59 depend directly or indirectly on claim 48, thus incorporate the limitations of base claim 48 and are patentable for at least the same reasons mentioned for claim 48. Therefore, Applicants' respectfully request reconsideration of the pending claims and withdrawal of the rejections.

***New Claims***

New claims 60-69 have been added. New independent claim 60 is directed to a composition wherein the genetically altered chondrocyte expresses erythropoietin or erythropoietin mimetibody. Dependent claims 61-67 are analogous to previously presented claims 49 and 54-59. Dependent claims 68-69 are directed to the genetically altered chondrocyte

isolated from cartilage tissue. Support for the new claims can be found throughout the specification, for example paragraphs [0030] and [0050] and examples 3 and 4 of the published application. No new matter has been added by the proposed new claims.

Also, new independent claim 60 would not be obvious over Glorioso et al. in view of Bartholomew et al. for the same reasons as given above. Namely, there are no teachings for a genetically altered chondrocyte cultured and modified to express a therapeutic agent at an ectopic site. Furthermore, applications and techniques used on stem cells, e.g. the mesenchymal stem cells of Bartholomew et al., are not applicable or obvious for use with differentiated cells, such as chondrocytes.

## CONCLUSION

Applicants submit that all claims are in condition for allowance, and allowance thereof is respectfully requested. If the Examiner believes that an interview would facilitate the resolution of any outstanding issues, the Examiner is kindly requested to contact the undersigned.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 141449, under Order No. 22956-225.

Dated: August 18, 2008

Respectfully submitted,

By_____
George Xixis
Registration No.: 38,664
NUTTER McCLENNEN & FISH LLP
World Trade Center West
155 Seaport Boulevard
Boston, Massachusetts 02210-2604
(617) 439-3746
Fax: (617) 310-9746
Attorney for Applicant

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# A Shift from Reversible to Irreversible X Inactivation Is Triggered during ES Cell Differentiation

Anton Wutz and Rudolf Jaenisch*
Whitehead Institute for Biomedical Research
9 Cambridge Center
Cambridge, Massachusetts 02142

## Summary

***Xist*** **is required for X inactivation. To study the initiation of X inactivation, we have generated a full-length mouse *Xist* cDNA transgene and an inducible expression system facilitating controlled *Xist* expression in ES cells and differentiated cultures. In ES cells, transgenic *Xist* RNA was stable and caused long-range transcriptional repression in *cis*. Repression was reversible and dependent on continued *Xist* expression in ES cells and early ES cell differentiation. By 72 hr of differentiation, inactivation became irreversible and independent of *Xist*. Upon differentiation, autosomal transgenes did not effect counting, but transgenic Xist RNA induced late replication and histone H4 hypoacetylation. *Xist* had to be activated within 48 hr of differentiation to effect silencing, suggesting that reversible repression by *Xist* is a required initiation step that might occur during normal X inactivation in female cells.**

## Introduction

X chromosome inactivation is an epigenetic event that results in the repression of genes on one of the two X chromosomes in female mammals. Thus, it compensates for a dosage imbalance arising from the unequal genetic constitution of XY males and XX females. X inactivation is initiated after implantation of the mouse embryo, and a counting mechanism ensures that all but one X chromosome become silent per diploid set of autosomes (reviewed in Heard et al., 1997). The process of random X inactivation can be recapitulated during the differentiation of mouse embryonic stem (ES) cells (Lee et al., 1996; Panning and Jaenisch, 1996; Penny et al., 1996; Clerc and Avner, 1998), providing for a tissue culture system to study the molecular mechanism of X inactivation. Inactivation starts at the X inactivation center (*Xic*) from where it spreads over the chromosome. The *Xist* gene is closely linked to the *Xic* and is transcribed exclusively from the inactive X chromosome (Brockdorff et al., 1991; Brown et al., 1991). *Xist* lacks any significant protein coding potential but has been shown to act as an RNA that associates with the inactive X chromosome (Clemson et al., 1996). Furthermore, the requirement of *Xist* for X inactivation has been demonstrated by loss-of-function experiments in mouse and ES cells (Penny et al., 1996; Marahrens et al., 1997). Studies in ES cells have also demonstrated that the function of the *Xic* can be reconstituted on autosomes by the insertion of YAC transgenes in multiple copies (Lee et al., 1996; Lee and Jaenisch, 1997; Heard et al., 1999a). *Xist* expression is the earliest known event in X inactivation, and it has been shown that the *Xist* gene is required for the initiation, but not for the maintenance of X inactivation (Brown and Willard, 1994; Csankovszki et al., 1999). *Xist* is regulated by a complex mechanism to ensure that all but one X chromosome per diploid chromosome set are inactivated. Hence, the presence of two functional *Xic*s is required to initiate X inactivation, and, if multiple *Xic*s are present, a choosing mechanism induces *Xist* expression from all but one *Xic*. While a number of recent studies (Panning et al., 1997; Sheardown et al., 1997; Clerc and Avner, 1998; Johnston et al., 1998; Marahrens et al., 1998; Lee and Lu, 1999) have focused on the regulation of *Xist*, little is known about the mechanism by which Xist RNA causes chromosome-wide repression.

To investigate the function of Xist RNA in chromosome-wide silencing, we have introduced an *Xist* cDNA transgene driven by an inducible promoter into mouse ES cells. We show that the 15 kb transgene has the ability to initiate chromosome-wide silencing when integrated in single copy on autosomes and on the X chromosome in ES cells. Transgenic *Xist* expression caused chromosomal late replication and histone H4 hypoacetylation during ES cell differentiation but did not trigger counting. We show that initiation of *Xist*-mediated silencing is restricted to early stages of differentiation when repression is reversible, and chromosomal inactivation becomes independent of *Xist* expression and irreversible at a subsequent stage of differentiation.

## Results

### Generation of ES Cells Carrying an Inducible *Xist* cDNA Transgene

The 15 kb mouse *Xist* cDNA was constructed from genomic fragments containing most of exon 1 and exon 7 and a cDNA fragment spanning the spliced region of *Xist* (Figure 1A). This cDNA corresponds to the somatic transcript of *Xist* and spans both somatic transcription initiation sites P1 and P2 (Johnston et al., 1998). In order to express *Xist* in a regulated manner, an inducible expression system was set up in J1 ES cells, a male 129 mouse ES cell line (Marahrens et al., 1997). The nls-rtTA cDNA (Gossen et al., 1995), encoding a doxycycline-inducible transcriptional activator, was introduced into the ubiquitously expressed *ROSA26* locus by homologous recombination (Soriano, 1999). The *Xist* cDNA transgene with a tetracycline-responsive promoter (Gossen et al., 1995) and a polyadenylation sequence was introduced into the inducible ES cells using a cotransfection strategy with a selectable puromycin resistance marker plasmid (*PGKpuro*). Puromycin-resistant ES colonies (96) were screened by RNA fluorescent in situ hybridization (FISH) for *Xist* expression in the presence of the inducer doxycycline. Of these, 15 expressed *Xist* at high

*To whom correspondence should be addressed (e-mail: jaenisch@wi.mit.edu).

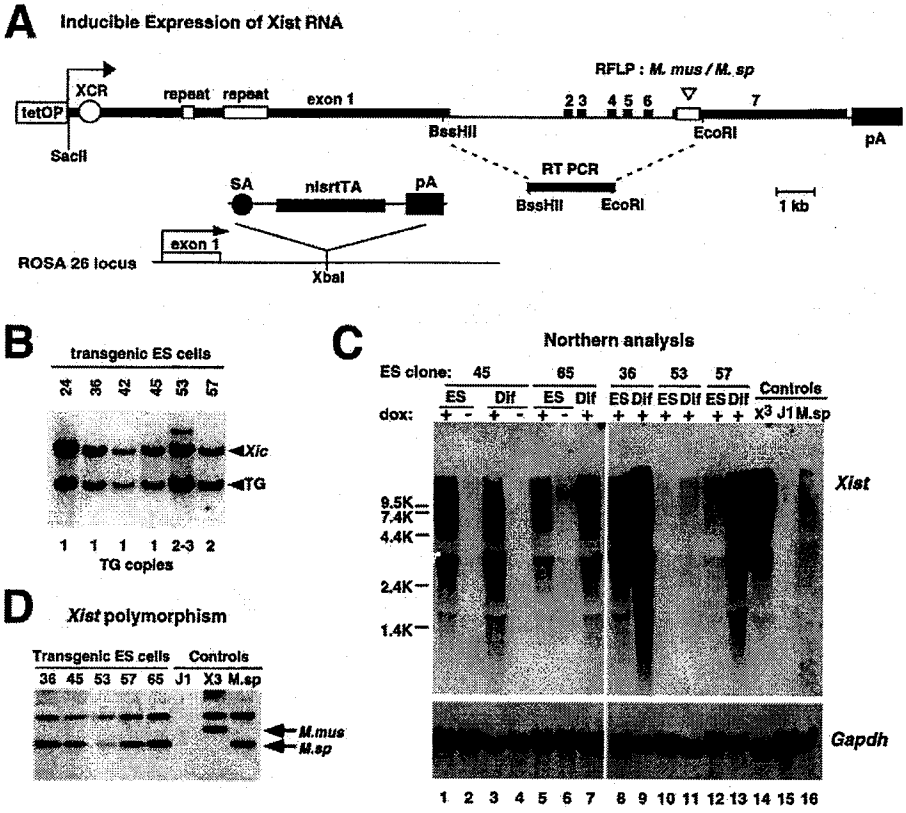


Figure 1. Inducible *Xist* Expression in ES Cells

(A) The *Xist* cDNA was cloned from genomic DNA fragments containing *Xist* exon 1 and exon 7 and an RT PCR product spanning the spliced region. Regulated *Xist* expression was achieved using J1 ES cells carrying a targeted insertion of the doxycycline-responsive transactivator nls-rtTA into the *ROSA26* locus. A *Mus spretus* repeat polymorphism distinguishes the transgene from the J1 ES cell *Mus musculus Xist* allele. Repeats (open boxes) and the XCR region (open circle) are indicated. pA, polyadenylation sequence; SA, splice acceptor.

(B) The copy number of the transgene (TG) was determined relative to the endogenous *Xist* gene (*Xic*) by Southern analysis of BamHI-digested DNA hybridized to the pX3 probe using a phosphoimager and the MacBAS V2.5 software (Fuji Photo Film Co., Ltd.).

(C) Northern analysis of 20 μg total RNA from transgenic ES cells hybridized to the pX3 probe, and *Gapdh* as a loading control. Induced transgenic ES cells (lanes 1, 5, 8, 10, and 12), induced differentiated cells (lanes 3, 7, 9, 11, and 13), uninduced samples (lanes 2, 4, and 6), controls: X3 female mouse somatic cells (lane 14), J1 ES cells (lane 14), *M. sp* adult kidney RNA (lane 16).

(D) Transgenic origin of the Xist RNA in differentiating ES cells was established by RT PCR analysis using MX1 and MX2 primers on RNA from transgenic ES cultures, which were differentiated for 4 days with retinoic acid. The PCR product was gel purified and digested with PstI, electrophoresed on a 2% agarose gel, blotted, and hybridized with the 800 bp PstI fragment containing the *M. mus* repeat. The positions of the polymorphic bands are indicated: transgene-derived Xist (*M. sp*), J1 ES cell–derived Xist (*M. mus*).

level in an inducible manner, and 7 of these were selected for further analysis. In all these lines, a single or a few copies of the transgene had integrated as determined by phosphoimager analysis of DNA blots (Figure 1B and Table 1). The transgene insertion sites were determined by DNA FISH on autosomes 1, 11, 12, 18, and the X chromosome (Table 1, Figure 3D, and data not shown). Analysis of RNA extracted from ES cells and differentiated cultures revealed that high molecular weight Xist RNA was produced when the cells were grown in the presence of the inducer doxycycline (Figure 1C). No or very little Xist RNA was detected in cultures grown without induction. In order to determine whether the Xist RNA originated from the transgene, a *Mus spretus* polymorphism in *Xist* exon 6 was used, which had been included in the cDNA transgene (Figure 1A). RT PCR analysis of *Xist* in RNA extracted from transgenic ES cells that had been differentiated for 4 days in the presence of doxycycline showed exclusive expression of the transgenic *Mus spretus* allele (Figure 1D). No *Mus musculus* allele was detected in differentiated parental male and transgenic ES cells. We conclude that doxycycline treatment induced full-length transgenic Xist RNA.

### Xist RNA Is Stable in Undifferentiated ES Cells and Leads to Reversible Long-Range Chromosomal Inactivation in *cis*

Using this inducible *Xist* expression system, we investigated the function of Xist RNA in undifferentiated ES cells. Northern analysis indicated that transgenic Xist RNA was abundant and stable in undifferentiated ES cells that had been grown in the presence of doxycycline (Figure 1C). RNA FISH analysis of transgenic ES cells using a Cy3-labeled *Xist* probe showed that the Xist transcript accumulated in the nucleus in large domains,

Table 1. Summary of Transgene Integrations

| ES Clone | TG Copies | Chromosomal Location | RNA Paints Dif | H4 Hypoacetylation Dif | Late Replication Dif | Repression of Genes | | Cell Death | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | ES | Dif | ES | Dif |
| 24 | 1 | X | ND | ND | ND | ND | ND | + | + |
| 36 | 1 | 11 | + | + | + | + | + | – | – |
| 42 | 1 | 11 | + | + | ND | + | ND | – | + |
| 45 | 1 | 18 | + | ND | ND | ND | ND | – | – |
| 53 | 2–3 | 12 | + | + | + | ND | ND | – | – |
| 57 | 2 | 1 | + | + | ND | ND | ND | – | – |
| 65 | 1 | X | + | – | ND | + | ND | + | + |

The chromosomal location, copy number, and information on effects of the transgene are listed.
+, effect was observed; –, effect was not observed; ND, no data were collected; ES, effect was observed in undifferentiated ES cells; Dif, effect was observed in differentiating cultures.

and transgenic Xist RNA associated with metaphase chromosomes (Figure 2A). To study the consequences of *Xist* expression in ES cells, we measured cell death in the presence and absence of doxycycline. *Xist* expression from autosomal transgenes had no obvious effect on cell proliferation and cell survival. However, induction of an X-linked transgene (clone 65) resulted in cell death (Figure 2E and Table 1), and a significant lag in proliferation of the culture was observed (Figure 2F). The transgene in this clone had integrated proximal from the *Xic* as demonstrated by DNA FISH (Figure 3D) and not affected the endogenous J1 ES cell *Xic* as judged by Southern analysis (data not shown). Repression of X-linked genes *Hprt* and *Pgk1* was observed on Northern blots (Figure 2G, *Hprt* and *Pgk1* are separated by 28 cM [Mouse Genome Database {MGD}, The Jackson Laboratory, Bar Harbor, Maine, on 10/23/1999; http://www.informatics.jax.org]). Expression of the *Utx* gene, which is known to escape X inactivation in mouse (Greenfield et al., 1998), and the autosomal genes *Gapdh*, *Oct4*, and *Dnmt* were not affected. This strongly suggested that long-range silencing in *cis* by transgenic *Xist* induction had led to inactivation of the X chromosome in ES cells causing lethality.

To investigate whether the cDNA transgene would cause repression of transcription on autosomes in ES cells, we studied clone 36 and 42 ES cells, where the *Xist* transgene had integrated into chromosome 11. Northern analysis demonstrated that expression of *Tk* and *U2af1-rs1* was repressed upon induction of *Xist* for 2 days compared to ES cells grown without induction (Figures 2C and 4C). *Tk* was repressed to 60% and 50%, and *U2af1-rs1* to 79% and 61% in clones 36 and 42, respectively. Taken together, these data indicated that *Xist* had affected expression of two autosomal genes in undifferentiated ES cells. The repression was specific to clones with *Xist* transgenes on chromosome 11 as indicated by expression of *Idh1* on chromosome 1 and *Gapdh* on chromosome 6, which were not affected (Figure 2C). Also, *Xist* expression from a transgene integrated on chromosome 12 in clone 53 ES cells had no effect on *Tk* or *U2af1-rs1* (Figure 2C). We further investigated the effect of transgenic *Xist* induction on the puromycin selection marker, which was introduced with the *Xist* transgene by cotransfection. We screened ES cell clones carrying autosomal transgene integrations for loss of puromycin resistance in the presence of doxycycline and identified clone 36 ES cells, which became sensitive to puromycin in the presence of the inducer (Figure 2B). This suggested that the *PGKpuro* gene was silenced by *Xist* activation in undifferentiated ES cells. Other transgenic ES cell clones did not show loss of puromycin resistance, which could be attributed to the presence of unlinked *PGKpuro* integrations. Alternatively, a linked *PGKpuro* gene could have been activated by the tetracycline-responsive promoter of the *Xist* cDNA transgene comparable to a bidirectional promoter (Baron et al., 1995). Indeed, some of the clones showed enhanced *PGKpuro* expression in the presence of doxycycline before and after differentiation explaining the failure to silence the gene.

In order to test whether X inactivation was reversible or irreversible in ES cells, we induced *Xist* expression in undifferentiated clone 36 ES cells with doxycycline for 4 days in the absence of puromycin. Cells were subsequently grown without inducer for 4 more days, and RNA was extracted and analyzed on Northern blots (Figure 2D). *PGKpuro* was efficiently repressed (over 90%) in cells grown in the presence of doxycycline (Figure 2D, lane 3), as compared to uninduced cultures (Figure 2D, lane 1). The cells that were induced and then grown without inducer had fully reactivated *PGKpuro* expression (greater than 95%, Figure 2D, lane 2) showing that repression was reversible.

We further measured the kinetic of *Xist*-mediated silencing in clone 36 and 65 ES cells. Transgenic *Xist* was induced in ES cells, and RNA was extracted 0, 6, 8,12, 22, and 30 hr thereafter. Northern analysis revealed that repression of puro and *Hprt* in clone 36 and 65, respectively, was observed after 22 hr of *Xist* induction (data not shown; *Gapdh* was used as a loading control). Xist RNA had accumulated significantly by 2 hr after doxycycline addition, and the steady-state level was reached after 4 to 6 hr as judged by RNA FISH and Northern analysis. Cell death resulting from X inactivation in clone 65 ES cells was detectable as early as 32 hr after addition of doxycycline. We measured the doubling time of ES cell cultures to 21.6 hr (1.11 cell doublings per 24 hr; standard deviation, 0.30; n = 10) and conclude that *Xist*-mediated silencing occurs within one cell division cycle.

Finally, we determined the half-life of Xist RNA in ES cells. *Xist* was induced in clone 36 ES cells for 3 days, doxycycline was washed out, and the cells were grown further in the presence of actinomycin D, an inhibitor of transcription. RNA was extracted after 0, 2, 4, 8, and 12 hr. By measuring Xist RNA abundance, we determined

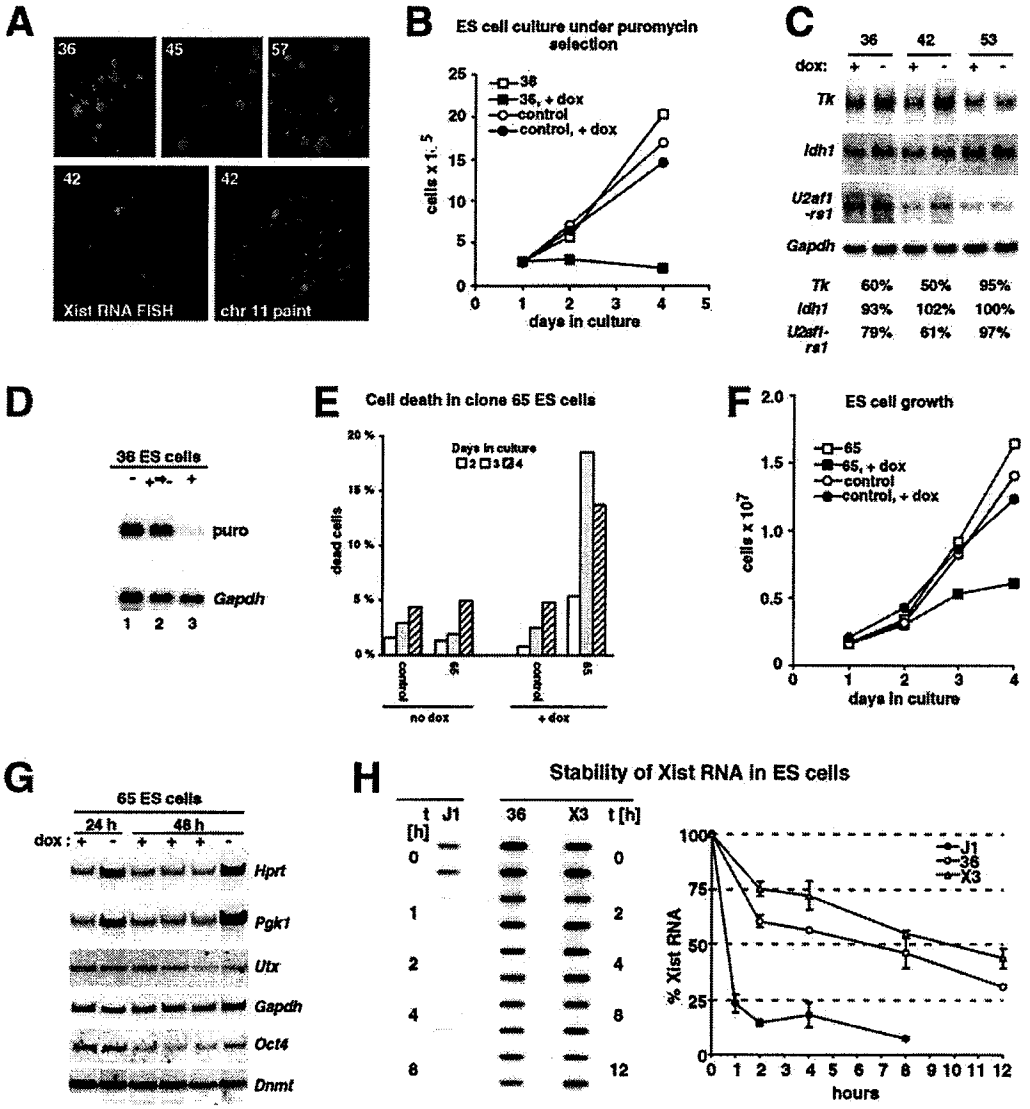


Figure 2. Ectopic X Inactivation in Undifferentiated ES Cells

(A) *Xist* expression in ES cells of clones 36, 45, and 57 grown on Roboz slides in the presence of 1 μg/ml doxycycline for 48 hr and fixed with paraformaldehyde was detected by RNA FISH using a Cy3-labeled pBP5.6 *Xist* probe (red), and chromosomal association of Xist RNA was detected in metaphase spreads of clone 42 ES cells. Subsequently, chromosome 11 was identified by DNA FISH using a paint probe.

(B) Proliferation of undifferentiated transgenic ES cells was measured in the presence of 2 μg/ml puromycin. The number of cells in the cultures was determined on day 1, 2, and 4. A reduction of cell number is observed in clone 36 upon induction with 1 μg/ml doxycycline. Control: clone 57 ES cells.

(C) Northern analysis of RNA extracted from transgenic ES cells grown for 2 days in the presence (+ dox) or in the absence (− dox) of doxycycline. *Tk* and *U2af1-rs1* are repressed by *Xist* induction in clone 36 and clone 42 ES cells, which carry an Xist transgene on chromosome 11. Expression is unaffected in clone 53 ES with a transgene insertion on chromosome 12. The level of expression (in percent) relative to the cells grown without doxycycline was determined using a Fuji phosphoimager and the MacBAS V2.5 software (Fuji Photo Film Co., Ltd.).

(D) Reversibility of repression of the *PGKpuro* marker (puro) in undifferentiated clone 36 ES cells (see text) was established by Northern analysis of RNA extracted from cells grown in the absence (lane 1) or presence of 1 μg/ml doxycycline for 4 days (lane 3) and from cells that were grown in the absence of inducer for 4 days after they had been induced for 4 days (lane 2).

(E–G) Analysis of X chromosomal transgene integration in ES clone 65.

(E) Cell death was measured in induced (dox) and noninduced undifferentiated ES cell cultures on day 2, 3, and 4 by Trypan blue staining of cells in the culture supernatant and is represented normalized to the number of cells in the culture. Control: J1 ES cells containing the doxycycline-responsive transactivator.

(F) The proliferation in these ES cell cultures was analyzed by life cell counting using Trypan blue dye exclusion.

(G) Northern analysis of RNA extracted from undifferentiated clone 65 ES cells grown in the presence of doxycycline (dox) for 24 hr or 48 hr or without doxycycline. Repression of the X-linked *Hprt* and *Pgk* genes is observed. *Utx*, which escapes X inactivation in mouse, *Gapdh* (located on chromosome 6), *Oct4* (chromosome 17), and *Dnmt* (chromosome 9) are unaffected.

(H) Stability of Xist RNA was measured. Clone 36 ES cells were grown in the presence of doxycycline for 3 days, then washed three times

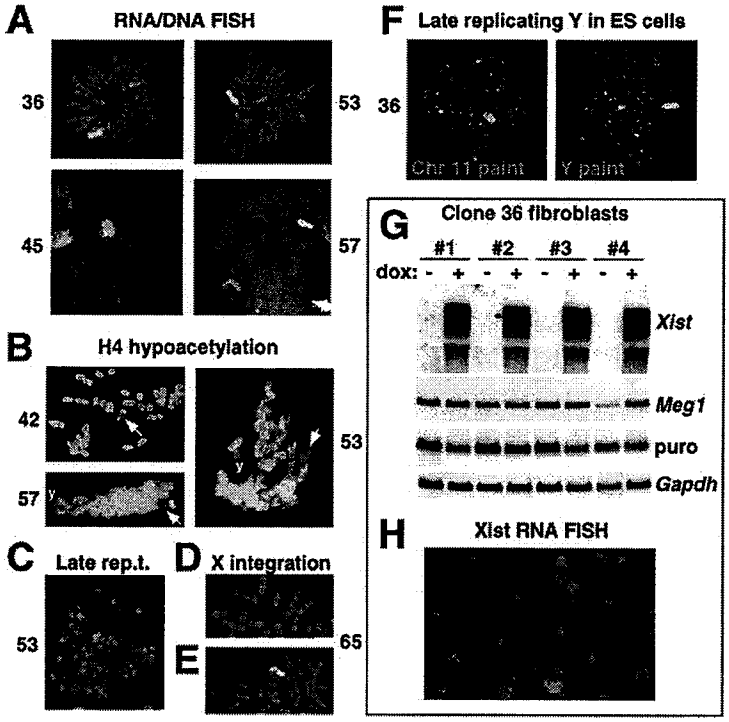


Figure 3. *Xist* Expression during ES Cell Differentiation

(A) Localization of Xist RNA in transgenic ES clones 36, 45, 53, and 57, which were differentiated for 6 days with retinoic acid, to metaphase chromosomes was observed using an RNA FISH pBP5.6 probe (red) and a DNA FISH X paint probe (green signal).

(B) Histone H4 acetylation was detected on metaphase spreads obtained from transgenic ES cells, which were differentiated for 6 days with retinoic acid, using an antiserum specific for acetylated histone H4 (green) and staining of the DNA with DAPI (blue).

(C) Late replication of chromosome 12 in clone 53 is demonstrated by BrdU incorporation (green signal) and a chromosome 12 specific DNA FISH probe (red signal). The transgene has been mapped to chromosome 12 in clone 53.

(D) X chromosomal integration of the transgene in clone 65 was established by DNA FISH using a fluorescein-labeled probe derived from a P1 clone spanning the *Xic* (green) and a Cy3-labeled *PGKpuro* probe (red). The result was confirmed with an *Xist* cDNA FISH probe in a separate experiment (data not shown).

(E) RNA FISH using a Cy3-labeled pX3 probe (red) to detect Xist RNA superimposed on DNA FISH using an X chromosome paint probe (green) shows that the X chromosome is partially painted by Xist RNA in clone 65.

(F) Late chromosomal replication in undifferentiated clone 36 ES cells, which have a transgene insertion on chromosome 11 and were grown in the presence of doxycycline for 3 days, was analyzed by BrdU incorporation (green signal). Chromosome 11 was identified using a DNA FISH paint probe (left), and the late replicating Y chromosome was identified using a Y paint probe (right).

(G) Northern analysis of RNA from fibroblasts that were established from four chimeric embryos (#1–#4) obtained from injection of clone 36 ES cells into blastocysts and were grown for 10 days in the presence or absence of doxycycline (see text).

(H) *Xist* expression in fibroblasts induced with doxycycline for 2 days was analyzed by RNA FISH using a Cy3-labeled BP6.5 *Xist* probe (red).

the half-life of transgenic Xist RNA to 6.5 hr (Figure 2H), and 6.2 hr in an independent experiment (data not shown), which is similar to the half-life of 6 hr previously published for female fibroblasts (Sheardown et al., 1997). *Xist* in control J1 ES cells had an apparent half-life of 30 min equal to published results (Sheardown et al., 1997) and 9.7 hr in X3 female mouse somatic cells.

### *Xist* Expression from Autosomal Transgenes Leads to Long-Range Chromosomal Silencing in Differentiating ES Cells

To investigate the effect of transgenic *Xist* expression on chromosome structure, we induced transgenic ES cells to differentiate with all-*trans*-retinoic acid in the absence of LIF (Hogan et al., 1994) for 6 days. Two-color FISH revealed that Xist RNA associated with mitotic chromosomes in metaphase preparations from transgenic ES cells that had been differentiated in the presence of doxycycline (Figure 3A). Subsequently, the X chromosome was identified using an X chromosome paint probe showing that Xist RNA localized to mitotic autosomes in four transgenic ES clones and covered a major part of the X chromosome in ES clone 65 (Figure 3E) with an X-linked transgene. The transgenic origin of the Xist RNA was established by allele-specific RT-PCR (Figure 1D and data not shown). To study the effect of transgenic *Xist* expression on chromatin, we assayed histone H4 acetylation and late replication, both hallmarks of the inactive X in female cells (Heard et al., 1997), in transgenic ES cells that were differentiated in the presence of doxycycline for 6 days. We determined H4 acetylation using an antiserum that specifically recognized acetylated histone H4 (Keohane et al., 1996). Underacetylated chromosomes were identified by absence of immunostaining in metaphases stained with DAPI identifying the DNA. ES clone 53 and 57 metaphases were obtained that contained a hypoacetylated Y chromosome and another hypoacetylated autosome (Figure 3B). In control experiments with cells differentiated without doxycycline, only hypoacetylated Y chromosomes were observed (data not shown). Similarly, a partially hypoacetylated chromosome was observed in ES clone 42 metaphases, suggesting that incomplete inactivation of the transgenic autosome had occurred.

with medium without doxycycline, and further incubated in the presence of 5 μg/ml actinomycin D for 0, 2, 4, 8, and 12 hr. RNA was extracted, and 5 μg was analyzed on a slot blot. X3 cells were analyzed in the same way. J1 ES cells were grown for 0, 1, 2, 4, and 8 hr in the presence of actinomycin D, and 10 μg was used for analysis. Signals were quantified using a phosphoimager and the MacBAS V2.5 software (Fuji Photo Film Co., Ltd.). Data for duplicate experiments were plotted.

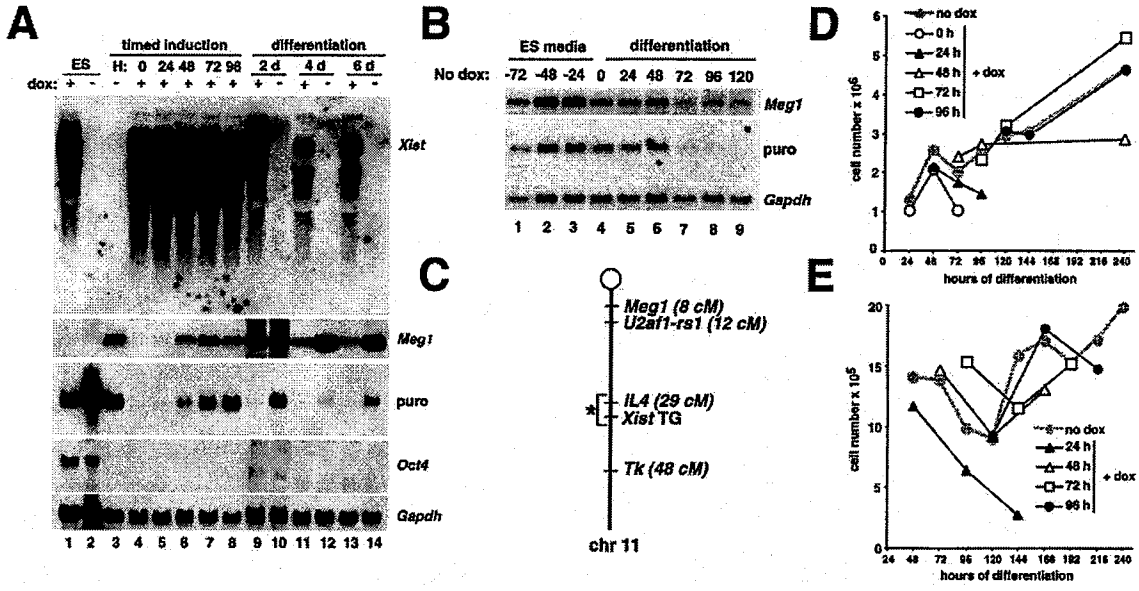


Figure 4. Initiation of Silencing in ES Cell Differentiation

(A) Analysis of ectopic inactivation of chromosome 11 in transgenic clone 36. Expression of *Xist*, *PGKpuro* (puro), *Meg1*, *Oct4*, and *Gapdh* was analyzed by Northern blot performed using 10 μg RNA from transgenic ES cells as follows. Lanes 1 and 2: undifferentiated ES cells induced for 4 days with 1 μg/ml doxycycline (lane 1) and without inducer (lane 2, note signal is overexposed). Lanes 3–8: ES cells were induced at 0, 24, 48, 72, and 96 hr after induction of differentiation with retinoic acid or uninduced (lane 3). RNA was extracted at 11 days after differentiation. Lanes 9–14: ES cells were differentiated in the presence (lanes 9, 11, and 13) or absence (lanes 10, 12, and 14) of doxycycline, and RNA was extracted 2 days, 4 days, and 6 days after differentiation.

(B) To test reversibility of silencing, clone 36 ES cells were grown in the presence of doxycycline for 4 days, and then the inducer was withdrawn at various time points before (−72, −48, and −24 hr) or after (0, 24, 48, 72, and 96 hr) cells were differentiated with retinoic acid. RNA was extracted at 12 days of differentiation and analyzed by Northern blot.

(C) Schematic representation of chromosome 11. Positions of *Meg1*, *U2af1-rs1*, *IL4*, *Tk*, *PGKpuro*, and the transgene in clone 36 are indicated (the genetic positions were retrieved from MGD on 11/16/99; http://www.informatics.jax.org). The asterisk indicates that the relative position of the transgene and the *IL4* gene are uncertain.

(D and E) Clone 65 ES cells were induced with 1 μg/ml doxycycline (dox) at 0, 24, 48, 72, and 96 hr after differentiation was initiated with retinoic acid, and cell counts were performed at several days following induction. A decrease in cell number relative to the uninduced control (no dox) is evidence for loss of cells due to initiation of ectopic inactivation of the X chromosome. Two independent experiments are shown (C and D).

To assay for late replicating chromatin in differentiated transgenic ES cell clones, bromo-deoxyuridine (BrdU) was incorporated into DNA of late S phase cells, and metaphases were obtained by arresting the cells with colcemid. Using a specific antiserum, BrdU incorporation in chromosomes was detected in metaphases obtained from transgenic ES cells that had been differentiated for 6 days in the presence of doxycycline. The identity of the late replicating chromosome was established for ES cell clone 53 by DNA FISH as chromosome 12 (Figure 3C), the same chromosome in which the transgene had integrated. Taken together, these observations indicated that transgene expression can lead to long-range silencing of autosomal chromatin in differentiated ES cells. This was in contrast to undifferentiated ES cells where no late replicating or hypoacetylated autosomes were observed after *Xist* activation. For example, metaphase spreads from undifferentiated clone 36 and 42 ES cells grown in the presence of doxycycline for 3 days were prepared after treatment of the cultures with BrdU and colcemid for 4 hr. We observed a late replicating Y chromosome in 29 out of 50 metaphases, but no late replicating chromosome 11 (Figure 3F). Similarly, a late replicating Y chromosome was observed in 27 out of 50 control metaphases in clone 36 ES cells grown in the absence of doxycycline and in 18 out of 50 metaphases of J1 ES cells. We further analyzed histone H4 acetylation in induced clone 36 ES cells and did not observe underacetylated chromosomes in 42 metaphase spreads analyzed (data not shown). Therefore, chromosomal late replication and histone hypoacetylation are effected by transgenic *Xist* expression after differentiation of ES cells, but silencing in ES cells is independent of these chromosomal modifications.

To further investigate the silencing of endogenous autosomal genes, we studied the expression of the imprinted genes *Meg1* and *U2af1-rs1* on chromosome 11, which are expressed from the maternally and paternally inherited chromosomes, respectively (Cattanach et al., 1998). Clone 36 ES cells with a transgene insertion on chromosome 11 (Table 1) were differentiated for 2, 4, and 6 days in the presence or absence of doxycycline, and RNA was analyzed on Northern blots. Induction of *Xist* had no significant effect on *U2af1-rs1* expression in differentiated ES cells (data not shown). However, repression of *Meg1* was observed by analysis of RNA extracted from transgenic clone 36 ES cells that were differentiated in the presence of doxycycline for 4 or

more days (Figure 4A, lanes 11–14) compatible with the view that the *Xist* transgene had integrated on and silenced the maternally inherited chromosome 11. *Meg1* expression was not detected in undifferentiated ES cells (Figure 4A, lanes 1 and 2) and initiated probably in a biallelic manner early after differentiation, as we were unable to detect significant repression by day 2 (Figure 4A, lanes 9–14). A switch from biallelic to monoallelic expression has been reported for other imprinted genes (Szabo and Mann, 1995). The transgene insertion site in clone 36 as determined by DNA FISH was localized close to *Il4* (chromosome 11, at 29.0 cM, MGD on 8/3/1999; http://www.informatics.jax.org) and is separated from *Meg1/Grb10* (at 8.0 cM, MGD on 8/3/1999; http://www.informatics.jax.org) by over 20 cM (equal to 40 Mb) chromosomal distance (Figure 4C). We conclude that *Xist* expression induced long-range silencing on chromosome 11.

### Xist RNA Can Initiate Chromosomal Silencing Only during Early ES Cell Differentiation

We were interested in if *Xist*-mediated silencing can be induced after ES cells were differentiated. In order to obtain a synchronous entry of the ES cells into differentiation, we induced differentiation with all-*trans*-retinoic acid (Hogan et al., 1994). We observed no obvious effect on proliferation when transgenic *Xist*-expressing ES cells carrying an autosomal transgene were differentiated. In contrast, induction of *Xist* expression in differentiating clones carrying X-linked transgenes (65 and 24) resulted in cell death depending on the time point of *Xist* activation. Cell death in clone 65 ES cells was seen only when transgenic *Xist* was activated within 24 hr or later (Figures 4D and 4E). Similarly, in embroid body cultures, no cell death was observed if *Xist* induction occurred 4 days after differentiation (data not shown). Induction of Xist RNA was monitored by RNA FISH and occurred with equal efficiency before and after differentiation. Using clone 36 cells with a transgene on chromosome 11 (Figure 4C), in which induction of transgenic *Xist* had no effect on proliferation, we investigated the window in ES cell differentiation for initiation of *Xist*-mediated silencing in detail using *PGKpuro* and *Meg1* as molecular markers. Differentiation of the cultures was monitored by *Oct4* expression, a marker for undifferentiated stem cells (Nichols et al., 1998). *Oct4* was detected in undifferentiated ES cells by Northern analysis but was no longer present after 48 hr of differentiation (Figure 4A, compare lanes 1 and 2 to 9 and 10). *Xist* was activated in clone 36 ES cells at 0, 24, 48, 72, and 96 hr after the ES cells had been induced to differentiate, and RNA was analyzed after 11 days of differentiation. Repression of *PGKpuro* and *Meg1* was observed only if doxycycline was added before 48 hr of differentiation (Figure 4A, lanes 3 to 8), confirming our findings with clone 65. We monitored *Xist* expression by Northern analysis (Figure 4A) and RNA FISH (data not shown) and found that induction of *Xist* expression occurred with equal efficiency throughout ES cell differentiation. We determined the percentage of proliferating cells throughout the differentiation process by measuring S phase entry by BrdU incorporation for 24 hr at every time point (Table 2), demonstrating that resistance to *Xist*-mediated silencing was not caused by cell cycle exit of the cells. Thus, our results suggest that initiation of *Xist*-mediated silencing was restricted to an early stage of ES cell differentiation.

Table 2. Proliferation of Differentiating ES Cell Cultures

| t[hr] | BrdU Positive | Total Cells | Percent of BrdU Positive |
|---|---|---|---|
| 24 | 312 | 318 | 98% |
| 48 | 244 | 254 | 96% |
| 72 | 399 | 496 | 80% |
| 96 | 353 | 484 | 73% |
| 120 | 240 | 343 | 70% |
| 144 | 224 | 391 | 57% |

Proliferation of differentiating ES cells was studied by S phase entry as determined by BrdU incorporation for 24 hr at the time points listed. BrdU incorporation was detected in spreads using a specific antiserum. Nuclei were counted after staining of DNA with DAPI.

To exclude artifacts of the ES cell culture system, we injected uninduced clone 36 ES cells into blastocysts and established fibroblast cultures from day 13.5 embryos. After elimination of host blastocyst–derived cells by puromycin selection, the cultures were split and grown with and without doxycycline for 10 days to allow for four cell doublings (Figure 3G). Northern analysis showed that *Xist* induction did not cause repression of *Meg1* and puro in the transgenic embryonic fibroblasts suggesting that initiation of X inactivation is also restricted in embryonic development. RNA FISH analysis showed the Xist RNA did accumulate in large nuclear clusters in fibroblasts induced with doxycycline (Figure 3H), and no Xist RNA signal was detected in uninduced cultures.

### Reversible *Xist*-Mediated Repression Becomes Irreversible during ES Cell Differentiation

To determine the reversibility and *Xist* dependence of silencing, we induced *Xist* expression in clone 36 ES cells for 4 days and then turned *Xist* off by doxycycline withdrawal at 72, 48, and 24 hr before shifting the cells to differentiation conditions (at 0 hr) and at 0, 24, 48, 72, 96, and 120 hr after induction of differentiation. In this way, we obtained cultures where *Xist* expression was turned off at various time points before and during differentiation. Only a weak remnant Xist RNA signal was detected by RNA FISH 12 hr after withdrawal of doxycycline, and the signal was completely extinguished after 24 hr (data not shown). Northern analysis of RNA extracted after 12 days of differentiation showed repression of *Meg1* and *PGKpuro* in cultures that had been grown in the presence of doxycycline up to 72 hr or more of differentiation (Figure 4B). However, when *Xist* was turned off prior to 72 hr of differentiation, *PGKpuro* and *Meg1* were efficiently reactivated, indicating that inactivation was reversible and dependent on *Xist* at this time. We conclude that *Xist*-mediated silencing became irreversible and independent of continuous *Xist* expression after differentiation of ES cells between 48 and 72 hr.

### Ectopic *Xist* Expression Does Not Activate the Endogenous *Xist*

In order to investigate whether the cDNA transgene would affect the endogenous *Xist*, we followed *Xist* expression through the differentiation process using the allele-specific RT PCR assay (Figure 1D and data not shown). Xist RNA was exclusively derived from the transgene, which was identified by a *Mus spretus* polymorphism (Figure 1A). Furthermore, two-color FISH analysis using a Cy3-labeled Xist RNA probe and a fluorescein-labeled X chromosome paint probe revealed a strong Xist RNA signal only in cells that were grown in the presence of doxycycline (data not shown). Colocalization of the Xist RNA with the *Xic* was not observed in ES clones with autosomal transgene integrations indicating that the endogenous *Xist* was not activated. We conclude that ES cells do not recognize the *Xist* cDNA transgene as an ectopic *Xic*, because no activation of the endogenous *Xist* had occurred.

## Discussion

We have established a tissue culture–based inducible expression system in ES cells to investigate the function of *Xist* RNA and to dissect the initiation phase of X inactivation. Our data demonstrate that Xist RNA produced from a 15 kb cDNA transgene was sufficient for chromosome-wide silencing in ES cells. All steps of *Xist*-mediated silencing were recapitulated by the transgene during ES cell differentiation; transgenic *Xist* associated in *cis* with autosomal chromatin, induced chromosomal late replication and histone H4 hypoacetylation, and caused long-range transcriptional repression. This suggests that transgenic Xist RNA can effect chromosomal repression similar to the inactive X in female cells.

### Initiation of X Inactivation in Undifferentiated ES Cells

In contrast to normal cells, Xist RNA derived from the cDNA transgene was stable in undifferentiated ES cells with a similar half-life of *Xist* in female somatic cells. Previously, it has been reported that *Xist* is expressed at a low level in ES cells and is unstable despite normal splicing (Panning et al., 1997; Sheardown et al., 1997). Stabilization of Xist upon differentiation of ES cells has been proposed as the regulatory step leading to accumulation of the RNA and subsequently chromosomal inactivation. The stability of the transgenic Xist RNA in ES cells may be due to the absence of 3′ or 5′ genomic sequences that are required for specific destabilization of the endogenous ES cell Xist transcript. Alternatively, stabilization of Xist RNA might depend on the amount of RNA present, and instability of Xist RNA in ES cells might arise as consequence of the low expression. Compatible with this view, the somatic *Xist* promoters P1 and P2 have been shown to be activated at the time of X inactivation in differentiating female ES cells (Johnston et al., 1998), and human Xist RNA produced from a YAC transgene has been reported to accumulate in the nucleus of mouse ES cells, suggesting that it was stable (Heard et al., 1999b). The rate of Xist RNA transcription was reported to be equal in ES cells and female somatic cells (Panning et al., 1997; Sheardown et al., 1997), leading to the conclusion that *Xist* upregulation upon differentiation was mediated by stabilization of the RNA. However, it is still conceivable that *Xist* expression is upregulated on the transcriptional level at the time when X inactivation is initiated in female ES cells and embryos, since neither undifferentiated ES cells nor somatic cells normally initiate X inactivation.

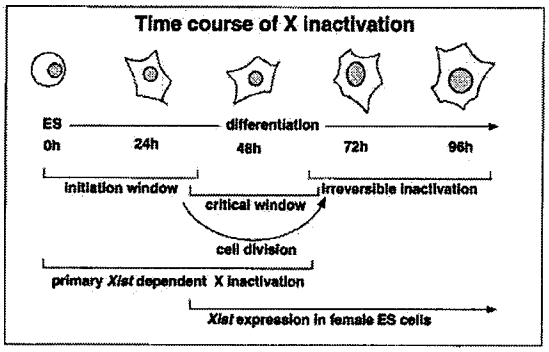


Figure 5. Time Course of *Xist*-Mediated Silencing

*Xist*-mediated silencing proceeds in two steps: (1) Primary inactivation depends on Xist and is reversible, and (2) irreversible maintenance of the inactive state. The initiation window specifies the time interval in which *Xist* is able to initiate repression. It is separated from irreversible inactivation by approximately one cell division, corresponding to the time required to effect primary inactivation. The primary inactivated chromosome is the substrate for irreversible inactivation. The timing of *Xist* expression in female ES cells is also indicated in the scheme.

Our experiments further demonstrate that induction of transgenic *Xist* caused long-range chromosomal repression on the X chromosome and on chromosome 11 in ES cells. The repression was limited to the chromosome harboring the *Xist* transgene, and, for the X chromosome, we were able to show that repression was specific for genes that are known to be susceptible to X inactivation, namely *Hprt* and *Pgk1*. *Utx*, which escapes X inactivation in mouse, was not affected by transgenic *Xist*, supporting our interpretation that transgenic *Xist*-mediated *cis* limited long-range repression in ES cells by a similar mechanism as X inactivation in female cells. Xist RNA accumulated in doxycycline-induced clone 36 ES cells to 86% of the level of X3 mouse female somatic cells, as measured by slot blot analysis (Figure 2H), indicating that inducible expression of *Xist* in ES cells was within the physiological range. In contrast to differentiated cells, *Xist*-mediated repression in ES cells was reversible and required continuous expression of *Xist*. We interpret this as the initial step of X inactivation that is followed by further modifications of chromatin during ES cell differentiation. Indeed, *Xist*-mediated silencing did not cause late replication or histone hypoacetylation of chromosomes in ES cells, suggesting that at this stage *Xist* acts on the promoter or transcription machinery and has no effect on replication or histone acetylation. We also show that repression is achieved within a single cell division from the induction of *Xist* expression.

### Cells Become Resistant to *Xist*-Mediated Silencing when X Inactivation Becomes Irreversible

We show that ectopic *Xist* expression did not initiate X inactivation after ES cells had been differentiated. This finding is in agreement with a report that mouse Xist RNA localizes to, but does not inactivate, the X chromosome in somatic cell lines that have been treated to reactivate *Xist* (Clemson et al., 1998). We observed resistance to *Xist*-mediated inactivation as early as 48 hr, equal to no more than two cell divisions, after the cells were shifted to differentiation conditions. X inactivation in female ES cells is initiated within the initiation window defined by our experiments (Figure 5), and we observed large clusters of Xist RNA by RNA FISH after 2 days of differentiation of female ES cells (data not shown) in agreement with the report that late replication of the inactive X chromosome can be observed at this time (Keohane et al., 1996).

Our experiments also reveal a transition from reversible to irreversible X inactivation in ES cell differentiation (Figure 5). *Xist* was no longer required for maintenance of the inactive state after 72 hr of differentiation, which is consistent with the observation that *Xist* is not needed for the maintenance of the inactive state in somatic cells (Brown and Willard, 1994; Csankovszki et al., 1999). In our experimental system, *Xist* expression had to be induced early enough to allow primary inactivation to take effect before silencing became irreversible and independent of *Xist* expression, suggesting that the initially inactivated chromosome is subjected to a "lock in" of the repressed state. Importantly, the initiation of *Xist*-mediated silencing had to occur at a time when repression was reversible. This shows that the initially reversible silencing in undifferentiated ES cells is required for later stages of the inactivation process to take effect, suggesting that it is part of normal X inactivation in female cells. We suggest that irreversibility of X inactivation and resistance to *Xist*-mediated silencing occur at the same time. This points to a transition in chromatin structure in the differentiation of ES cells and may also involve methylation of the inactive X chromosome (Panning and Jaenisch, 1996). Furthermore, our observation is consistent with the report that chromatin undergoes a change during ES cell differentiation, indicated by the histone H4 hypoacetylation of centromeres, Y chromosomes, and inactive X chromosomes around this time (Keohane et al., 1996).

### Xist RNA and the Regulation of X Inactivation

Present models of X inactivation predict that *Xist* interacts with protein factors and implicate these factors in the regulation of X inactivation (Panning et al., 1997; Johnston et al., 1998; Lee and Lu, 1999). We suggest that upregulation of *Xist* transcription is the trigger for initiation of silencing and demonstrate that it is sufficient. Our results are consistent with the notion that Xist RNA itself is the factor that determines primary X inactivation and all other factors required for the initiation of silencing are present in ES cells. Xist RNA is able to spread and silence in *cis* independent of flanking and intronic DNA sequences. We extend previous observations that Xist RNA produced from multicopy YAC or cosmid transgenes can associate with autosomal chromatin (Lee et al., 1996; Herzing et al., 1997; Lee and Jaenisch, 1997; Heard et al., 1999a) by using single copy transgenes containing exclusively cDNA sequences. We further demonstrate that all steps of *Xist*-mediated silencing occur during differentiation of transgenic male ES cells independent of counting. This suggests that no factors other than Xist RNA itself are regulated by the counting machinery to achieve X inactivation. However, our data show that factors involved in the lock in of X inactivation are regulated by ES cell differentiation.

## Experimental Procedures

### Plasmid Construction and Probes

For the ROSA26 targeting vector, a 4 kb XhoI fragment from pSAβgeo (Soriano, 1999) was ligated into a derivative of pBluescript (Stratagene) lacking sites from ClaI to SacI. A 1.5 kb EcoRI-BamHI fragment from pUHD 172–1neo (Gossen et al., 1995) was ligated into the SacI-ClaI-digested plasmid. The 1.5 kb XhoI insert was cloned together with a 1.8 kb XbaI-SalI fragment from PGKneo (Marahrens et al., 1997) into the XbaI site of ROSA26–1 (Soriano, 1999), giving plasmid R26/N-nlsrtTA. For the *Xist* cDNA, a 5 kb PstI fragment from a 129 mouse BAC clone containing *Xist* exon 7 was ligated into pBluescript. A 3.8 kb PstI-PvuII fragment was cloned into PstI-EcoRV cut pBluescript, giving pEP3.8. A 2.1 kb RT PCR product using primers MX1 (5′-GGTCTTTTTTGGGAGCTATTGTGTATGAG-3′), and MX2 (5′-AACAAGAATTCACTAAGGATAGAAGCAGC-3′) on *Mus spretus* brain cDNA primed with MXRT (5′-CACTTGAATCCTGACC GGACGGAATGATG-3′) was cloned in pGEM-T vector (Promega). The SacII-EcoRI fragment was inserted into pEP3.8, giving pBP5.6. A 9.2 kb SacII-BssHII fragment containing *Xist* exon 1, isolated from a 129 mouse BAC clone, was inserted into pBP5.6, giving pXist. The 15 kb SacII-SalI fragment was cloned into pUHG 17–1 (Gossen et al., 1995), giving pCMV-Xist-PA. A 452 bp XhoI-HindIII fragment from ptetSPLICE (Gossen et al., 1995) was inserted into XhoI-HindIII-digested pUHG 17–1. A 1.1 kb PvuI-SacII fragment was inserted into PvuI-SacII-digested pCMV-Xist-PA, giving ptetOP-Xist-PA. Probe BP5.6 is the BssHII-ClaI insert of pBP5.6, and pX3 is a 3.3 kb EcoRI-ClaI fragment of pBP5.6. The *Hprt* probe is a 696 bp PCR product using the primers 5′-ATGCCCAGCGTCGTGATTAG-3′ and 5′-TGG CAACATCAACAGGACTC-3′, *Pgk1* (619 bp) using 5′-GAAGGGAAG GGAAAAGATGC-3′ and 5′-CAATCTGCTTAGCTCGACCC-3′, *U2af1-rs1* (661 bp) using 5′-GTACTGCGGATAGCCAGGTAAC-3′ and 5′-CACCAGCCTACAAAGCTTGAAC-3′, *Tk* (420 bp) using 5′-CTTTCG GCAGCATCTTGAACCT-3′ and 5′-CAGCGAGTGGCAGCCTGTTC-3′, and *Idh1* (220 bp) using 5′-GCACTGTCTTCAGGGAAGCTAT-3′ and 5′-CCTTCTGAGTTCCATCTTTTGG-3′. *Gapdh* was detected with a 850 bp XbaI-AseI fragment of the cDNA, *Dnmt* with a 500 bp PstI fragment of the cDNA, *Oct4* with a 2.1 kb genomic HindIII fragment containing exon 1, and *Utx* with a 1.2 kb NotI fragment of plasmid 1.1N (Greenfield et al., 1998).

### Cell Culture and Generation of Transgenic ES Cells

J1 ES cells were grown on mouse embryonic fibroblasts in DMEM (GIBCO), 15% fetal calf serum (FCS, Hyclone), and 250 U LIF/ml (Marahrens et al., 1997) or 1000 U/ml when addressing the effect of *Xist* on undifferentiated ES cells. Differentiation of ES cells was induced in ES media without LIF with 100 nM all-*trans*-retinoic acid, or embroid bodies were aggregated in hanging drop cultures in ES media without LIF for 2 days, then transferred to suspension cultures in bacterial plates (Fischer Scientific). Doxycycline-inducible ES cells were generated by transfecting J1 cells with 30 μg ClaI linearized R26/N-nlsrtTA plasmid using a Bio-Rad Genepulser set at 25 μF and 400V. After selection with 0.3 mg/ml G418 (GIBCO) for 9 days, colonies were screened by Southern analysis of HindIII-digested DNA hybridized with a probe specific for *ROSA26* exon 1 (Soriano, 1999). A 3.8 kb fragment identified the targeted allele (wild-type at 4.4 kb). The cells were tested for inducibility by transient transfection of the pBI-EGFP-luc (Clontech) and a CMVβgal control plasmid. Luciferase activity was measured in cultures grown with or without doxycycline using Reporter lysis buffer (Promega) and the Luciferase Assay Reagent (Promega) and was normalized to

β-galactosidase activity determined with the luminescent β-galactosidase detection kit II (Clontech). A 166-fold induction was measured (standard deviation, 54; n = 3). *Xist* transgenic ES cells were generated by electroporation of 50 μg ptetOP-Xist-PA and 5 μg *PGKpuro* plasmid, both PvuI linearized, into the inducible J1 ES cells. Puromycin (2 μg/ml) -resistant colonies were split into 96-well plates (Falcon) and 10-well ROBOZ slides (Cellpoint). The latter were analyzed by RNA FISH using a Cy3-labeled (Amersham) BP5.6 probe after 24 hr induction with 1 μg/ml doxycycline. Chimeras were generated by injection of ES cells into Balb/c blastocysts as described (Hogan et al., 1994). X3 cells (C. Brisken, personal communication), a mouse mammary cell line with a stable karyotype and two inactive X chromosomes, were grown in DMEM with 10% FCS.

### Preparation of Cells for Cytogenetic Analysis, RNA and DNA FISH, and Histone H4 Acetylation

For FISH, cells were grown on ROBOZ or cytospun onto *Superfrost Plus* slides (VWR Scientific), extracted with cytoskeletal buffer, and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS. Spreads were prepared from cells fixed with methanol-acetic acid (3:1). RNA and DNA FISH was performed as described (Panning and Jaenisch, 1996; Csankovszki et al., 1999). For late replication assays, cells were grown for 4–5 hr in the presence of 1 mM BrdU and arrested with colcemid (GIBCO), and spreads were prepared. BrdU was detected after denaturation for DNA FISH using a mouse anti-BrdU antibody (Becton Dickinson) and a fluorescein-conjugated anti-mouse antibody (Vector). Histone H4 acetylation was detected on spreads of unfixed cells as described (Keohane et al., 1996).

### RNA Analysis

RNA was prepared using the guanidiniumthiocyanate/CsCl protocol (Sambrook et al., 1989) or Trizol reagent (GIBCO). Northern analysis was performed as described (Sambrook et al., 1989). For RT-PCR analysis, 10 μg total RNA were treated with RNase free DNase (Promega) and reverse transcribed using Superscript II (GIBCO).

### Acknowledgments

We are grateful to Philippe Soriano for providing the ROSA26 targeting vector; Andy Greenfield for the mouse *Utx* probe; Cathrin Brisken for the X3 cells; Györgyi Csankovszki and B. Panning for discussions on FISH; Jessica Dausmann and Jan Loring for technical assistance; and Györgyi Csankovszki, Ted Rasmussen, and Britt Rideout for critically reading the manuscript. This work was supported by an Erwin Schrödinger fellowship from the Austrian Science Fund and a long-term fellowship from the Human Frontiers Science Program Organization awarded to A. W. and by NIH grant #R35-CA44339.

Received August 24, 1999; revised January 27, 2000.

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